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EFFECTS OF ACUTE STRESS ON GENE EXPRESSION OF SPLENIC CATECHOLAMINE BIOSYNTHETIC ENZYMES IN CHRONICALLY STRESSED RATS

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Abstract - The aim of this study was to examine how acute immobilization stress affects the concentrations of catecholamines in the plasma and the expression of the splenic catecholamine biosynthetic enzymes tyrosine hydroxylase (TH), dopamine-ß-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) in chronically socially isolated rats. We found that acute immobilization increases the plasma catecholamine levels and splenic PNMT protein levels in chronically socially isolated rats. These results show that acute stress of chronically stressed animals activates the sympatho-adrenomedullary system and increases synthesis of splenic PNMT by 37%, both of which can modulate the immune function.

Key words: Stress, chronic social isolation, acute immobilization, catecholamine, spleen, rats, qRT-PCR

INTRODUCTION

Most of the primary and secondary lymphoid tissues (including the spleen) receive dense sympathetic nervous system (SNS) innervations. Adrenaline (A) and noradrenaline (NA) produced by these sympathetic nerves may modulate cellular function by acting on the $ß_2$ adrenergic receptors of B and Th1 cells (Sanders et al., 1997). It is known that the optimal NA concentration is necessary for peak antibody responses to a T-cell dependent antigen (Flesner, 2000). Tail shock stress increases circulating NA (*via* tissue spillover) and drives the SNS to the extent that the splenic NA concentration is depleted or reduced below control levels for several hours after stressor termination (Greenwood et al., 2003). These findings suggest that stress can modulate immune function and that SNS may be involved in this immunomodulation. In addition, the literature data have shown that catecholamine biosynthetic enzymes are expressed in lymphoid tissues, such as the spleen and thymus (Andreassi et al., 1998; Warthan et al., 2002). Catecholamine biosynthetic enzymes include tyrosine hydroxylase (TH), a "rate-limiting" enzyme of catecholamines biosynthesis; dopamine-β-hydroxylase (DBH) that converts dopamine (DA) into NA, and phenylethanolamine N-methyltransferase (PNMT) that catalyzes the conversion of NA to A.

Chronic social isolation (CSI) represents a very strong stressor that can induce neuroendocrine and immune changes in animals. One of the key questions in stress research is how the same stressor can elicit a variant or altered response depending on prior experience with the current or different stressor. Weiss et al. (2004) reported that socially isolated rats

had enhanced responses to stressors. Immobilization is frequently used as an additional acute stressor and is considered as one of the most intensive stressors that significantly changes gene expression (Kvetnansky et al., 2009). Very little is known about catecholamine synthesis in the spleen after the exposure of chronically socially isolated rats to additional acute immobilization stress. The response to novel additional acute immobilization stress might reveal the detailed mechanisms underlying the gene expression of catecholamine biosynthetic enzymes in the spleen and the catecholamine impact on immune functions in conditions of chronic stress. In this study we examined changes in the gene expression (mRNA and protein levels) of catecholamine biosynthetic enzyme (TH, DBH and PNMT) levels in the spleen, as well as the concentrations of NA and A in the plasma after the exposure of chronically socially isolated rats (CSI) to additional acute immobilization stress (CSI+IMM).

MATERIALS AND METHODS

Animals

Wistar male rats (11 weeks old) were used. Animals were kept under standard laboratory conditions with water and food *ad libitum*, three to four per cage. Care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the "Vinča" Institute of Nuclear Sciences, Belgrade, Serbia, which are in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health, Bethesda, MD, U.S.A. The animals were divided into two groups. The **CSI group** (n=10) consisted of animals that were subjected to social isolation with a single animal per cage for 12 weeks. The **CSI+IMM group** (n=20) consisted of animals exposed to CSI stress for a period of 12 weeks, and after chronic stress these animals were exposed to additional acute IMM stress for a period of 2 h. Immobilization stress was provoked as described by Kvetnansky and Mikulaj (1970). Group CSI+IMM contained 20 animals. This group was divided into two subgroups (n=10), because we examined changes in catecholamine biosynthetic enzymes in two different time periods after the cessation of immobilization. The animals were sacrificed after chronic stress, 3 h and 22 h after the acute immobilization. Literature data show that in this period changes in the gene expression of catecholamine biosynthetic enzymes are to be expected (Kvetnansky et al., 2009; Wong and Tank, 2007). Samples of blood were collected and the spleens were rapidly dissected, frozen in liquid nitrogen and stored at -70°C until analyzed.

RNA isolation and cDNA synthesis

Total RNAs were isolated from 0.08 g of spleen tissues using TRIZOL reagent (Invitrogen, USA). After the isolation of mRNA, DNase treatment was applied with DNase I (Fermentas, Lithuania). The concentration of total mRNA was measured in triplicate on a spectrophotometer (Pharmacia, GeneQuantII Bio-Tech, USA). The quality of the mRNA was checked on agarose gel. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (Amersham Biosciences, UK) and pd $(N)_6$ Random Hexamer (Amersham Biosciences, UK) primer according to the manufacturer's protocol. 12µl of the sample that contained 1500ng mRNA was incubated 10 min at 65°C. Then a 21µl reverse transcriptase with pd (N) 6 primer (final 0.2µg) was added per sample and incubated 1 h at 37°C.

Real-time RT-PCR

TH, DBH and PNMT mRNA levels were quantified by quantitative real-time RT-PCR. TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for TH (Rn00562500_m1), DBH (Rn00565819_m1) and PNMT (Rn01495589_g1). The gene expression assays contained primers for the amplification of the target gene and the TaqMan MGB (Minor Groove Binder) probe 6-FAM dye-labeled for the quantification. Reactions were performed in a 25 µL reaction mixture containing 1x TaqMan Universal Master Mix with AmpErase UNG, 1x Assay Mix (Applied Biosystems, USA) and cDNA template (10ng of RNA converted to cDNA). PCR was carried out in the

ABI Prism 7000 Sequence Detection System at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The experimental threshold was calculated based on the mean baseline fluorescence signal from cycles 3 to 15 plus 10 standard deviations. The point at which the amplification plot crosses this threshold defined as Ct, represents the cycle number at this point and it is inversely proportional to the number of target copies present in the initial sample. Each sample was run in triplicate and the mean value of each Ct triplicate was used for further calculations. The reference gene (endogenous control) was included in each analysis to correct for the differences in inter-assay amplification efficiency; all transcripts were normalized to cyclophyline A (Rn00690933_m1) expression. The reaction mixture for endogenous control gene amplification consisted of 1x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems, USA), 1x Assay (6-FAM dye-labeled MGB probes) and cDNA (10 ng of RNA converted to cDNA). The levels of expression of cyclophyline A in samples under different treatments were checked by additional experiments that confirmed that the chosen reference gene was not regulated. Before quantification, validation experiments were performed to determine the similar amplification efficiency of the endogenous control and each target gene. We tested cyclophyline A and demonstrated that its efficiency of amplification was approximately equal to all assays used for the target genes. Briefly, serial dilutions of cDNA were prepared and amplified by real-time PCR using specific primers and fluorogenic probes for the target and endogenous control gene.

Quantification was done using the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (2001). The results obtained were analyzed by the RQ Study Add On software for 7000 v 1.1 SDS instrument (ABI Prism Sequence Detection System, Applied Biosystems, USA) with a confidence level of 95% ($P<0.05$). The relative expression of the target gene was normalized to cyclophyline A and expressed in relation to the calibrator, i.e. the control sample. Due to individual differences among animals, one sample from the control group with the expression value closest to the mean of all samples in this group and with the lowest measurement error was chosen as a calibrator. The results are reported as a fold change relative to the calibrator and normalized to cyclophyline A using the equation: $N_{sample} = 2^{-\Delta\Delta Ct}$.

Western blot analysis

The TH, DBH and PNMT proteins were assayed by Western blot analysis. The spleens were homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Protein concentration was determined using the BCA method (Pierce, USA), described by Stich (1990). The samples were boiled in denaturing buffer according to Laemmli (1970), for 5 min at 95°C. Fifteen micrograms of protein extract from the spleen was separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a supported nitrocellulose membrane (Hybond™ C Extra, Amersham Biosciences, UK). The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline-Tween (TBST). All following washes and antibody incubations were also carried out in TBS-T at room temperature on a shaker. Protein molecular mass standards (PageRuler™ Plus Prestained Protein Ladder, Fermentas) were used for calibration. The antibodies used for the quantification of specific proteins were as follows: for TH the monoclonal primary antibody against mouse TH (monoclonal antibody against TH from mouse-mouse hybrid cells, clone 2/40/15, dilution 1:5000, Chemicon International, USA); for DBH the anti-dopamine-ß hydroxylase (N-terminal) antibody, sheep (dilution 1:5000, Sigma, USA), for PNMT the polyclonal ant-PNMT primary antibody, rabbit (dilution 1:1000, Protos Biotech Corporation, USA), and for β-actin the rabbit polyclonal anti-β-actin (ab8227, dilution 1:5000, Abcam, USA). After washing, the membranes were incubated in the secondary anti-mouse, anti-rabbit (dilution 1:5000, Amersham ECL™ Western Blotting Analysis System, UK) and anti-sheep (dilution 1:5000, Calbiochem, Germany) antibodies conjugated to horseradish peroxidase. A secondary antibody was then visualized by the Western blotting enhanced chemiluminescent detection system (ECL, Amersham Biosciences, UK). The membranes were exposed to ECL film (Amersham

Biosciences, UK). Densitometry of protein bands on the ECL film was performed by Image J analysis PC software. The result was expressed in arbitrary units normalized in relation to β actin.

Catecholamine measurement

Plasma catecholamines were measured by the standard radioenzymatic assay described previously by Peuler and Johnson (1977), and the values were expressed as pg/ml plasma. The catecholamines present in the plasma aliquots were converted to their labeled O-methylated derivatives by S-(3H) adenosylmethionine (Lacomed, Czech Republic) and the lyophilized catechol-O-methyl transferase isolated from the rat liver. The O-methylated derivatives of the amines were then extracted along with unlabeled carrier compounds. The O-methylated derivatives were oxidized to ^{3}H-vanilline. Radioactivity was measured with a toluene-based scintillation liquid and with an LKB-Wallac model 1219 scintillation counter (Stockholm, Sweden) at 40% efficiency for tritium. The range of measurement was Window 1 5-320, sensitivity was 20 CPM, and the inter-assay was less than 10%.

Data analysis

The data are presented as means ± S.E.M. differences in the gene expression (mRNA and protein levels) of the catecholamine biosynthetic enzymes (TH, DBH and PNMT) and the concentration of NA and A in the plasma were analyzed by one-way ANOVA. The effects of additional acute immobilization of chronically socially isolated animals (CSI+IMM) compared to chronically socially isolated animals (CSI), were tested by Tukey's post-hoc test.

Correlations of the mRNA levels, protein levels and hormone levels were analyzed by the Spearman test, using Sigma Plot v10.0 (with Sigma Stat integration).

Statistical significance (p) was set to 0.05 statistical power (1-ß) exceeding 85%. The statistical power confirmed that the number of animals (n=10) was sufficient for this experiment. A reliability test was designed so we did three repeated measurements of the level of gene expression of TH, DBH and PNMT. The calculated value of the ICCR test of >0.85 was considered to be satisfactory and it proves the reliability of the applied methods. Statistical analysis was carried out using the SPSS.

RESULTS

One-way ANOVA analysis revealed significant changes in the NA ($F=15.38$; $p<0.05$) and A ($F=19.72$; $p<0.05$) plasma concentrations and PNMT ($F=14.23$; $p<0.05$) protein level in the spleen under the examined treatments.

The exposure of socially isolated animals to acute immobilization led to a significant 49% increase in the NA concentration ($p<0.05$, Tukey's test, Fig. 1) and a 50% increase in the A concentration ($p<0.05$, Tukey's test, Fig. 1) in the plasma 22 h after the cessation of immobilization. The additional acute immobilization of chronically socially isolated animals increased the splenic PNMT protein level by 37% ($p<0.05$, Tukey's test, Fig. 3) 22 h after the cessation of immobilization, whereas the mRNA level of this enzyme was unchanged 3 h after the cessation of immobilization (Fig. 2). As shown in Figs. 2 and 3, additional acute immobilization of chronically socially isolated animals did not affect the levels of TH and DBH gene expression in the spleen.

In the CSI animals we recorded significant positive correlation between the PNMT protein level in the spleen and the A level in the plasma 22 h after immobilization (Spearman, $\rho=0.867$, $P<0.0005$, Fig. 4).

DISCUSSION

In this work, we recorded that the acute immobilization stressor triggers an exaggerated elevation in plasma catecholamines in animals previously exposed to chronic social isolation, even 22 h after the exposure. This could mean that prior experience may condition the physiological systems to "expect"

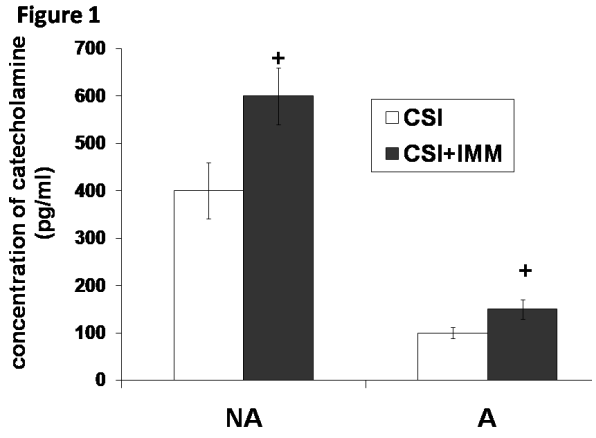

Fig. 1. Effects of additional acute immobilization stress on the concentration of noradrenaline (NA) and adrenaline (A) in the plasma of chronically socially isolated rats 22 h after immobilization. The values are means ± S.E.M. of 10 rats. Statistical significance: +p<0.05 animals exposed to additional acute 2 h immobilization stress after chronic social isolation (CSI+IMM) *vs.* animals exposed to chronic social isolation (CSI).

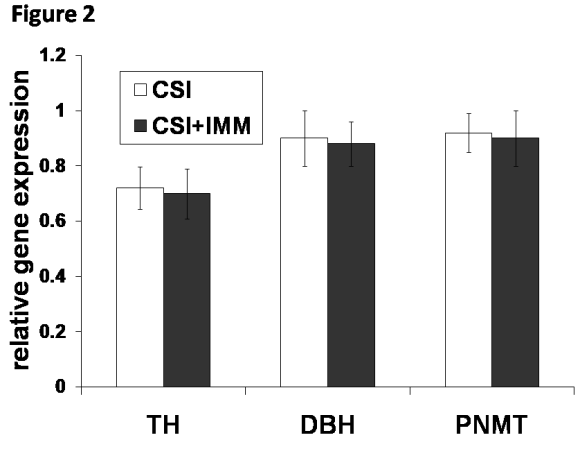

Fig. 2. Effects of additional acute immobilization stress on tyrosine hydroxylase (TH), dopamine-ß-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) mRNA levels in the spleen of chronically socially isolated rats 3h after immobilization. The values are means ± S.E.M. of 10 rats. The final result was expressed as fold change relative to the calibrator and normalized to cyclophyline A.

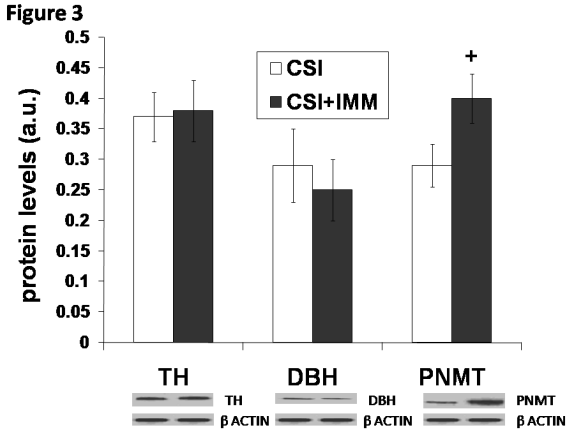

Fig. 3. Effects of additional acute immobilization stress on tyrosine hydroxylase (TH), dopamine-ß-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT) protein levels in the spleen of chronically socially isolated rats 22h after immobilization. The values are means ±S.E.M. of 10 rats. Statistical significance: +p<0.05 animals exposed to additional acute 2h immobilization stress after chronic social isolation (CSI+IMM) *vs.* animals exposed to chronic social isolation (CSI). The result was expressed in arbitrary units normalized in relation to β actin. (A) Distribution of TH, DBH, PNMT and ß actin proteins in the spleen of animals exposed to CSI [I] and animals exposed to CSI+IMM (22h after immobilization) [II].

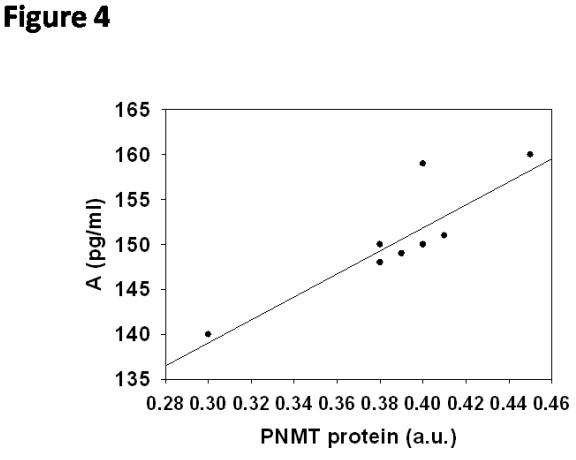

Figure 4. The correlation between PNMT protein level in the spleen and A level in the plasma of animals exposed to additional acute 2h immobilization stress after chronic social isolation (Spearman).

a problem and therefore be more ready to respond to a novel additional acute stressor. The readiness of the organism exposed long-term to a homotypic stressor to respond to a heterotypic stressor by an exaggerated activation of catecholamines is considered an important adaptive phenomenon of

the sympathetic-adrenomedullary system in rats (Kvetnansky et al., 2009). Increased concentration of catecholamines in the plasma can modulate the immune functions, which is in accordance with the reports of Shao et al. (2003) who found that an increase in plasma NA levels correlates with a decrease in antibody levels.

Stress-induced splenic NA depletion results in antibody suppression (Shao et al., 2003). This prompted us to investigate changes in catecholamine synthesis in the spleen after exposure of chronically stressed rats to acute immobilization stress. Detection of a regulatory mechanism for catecholamine synthesis in the spleen under conditions provoked by the additional acute immobilization of chronically stressed animals is exceptionally relevant in stress biology because of the significant role of catecholamines in modulation immune function. The results of this work are that CSI+IMM treatment does not affect the synthesis of splenic noradrenaline biosynthetic enzymes (TH and DBH), which indicates the absence of *de novo* synthesis of NA and suggests a probable exogenous source of NA in the spleen. A significant result in this study is that although PNMT mRNA is unchanged after CSI+IMM treatment regimes, this treatment may lead to a continuous accumulation of PNMT proteins as an adaptation to the applied stress. This adaptive response is necessary to maintain the biosynthetic capacity of A in the spleen during periods of sustained A secretion. Significant positive correlation between the levels of PNMT protein in the spleen and A in the plasma of animals exposed to CSI+IMM support this. Therefore, during CSI+IMM treatment the increased synthesis of splenic PNMT protein affects the sustained increase of A. Our data raise the possibility that the spleen converts the sympathetic neurotransmitter NA to A, and confirm that the SNS is one of the major pathways involved in immune-neuroendocrine interactions. Because A is more potent than NA as a ß$_2$-agonist (Lands et al., 1967) and has actions on the immune system not observed with NA (Jetschmann et al., 1997), the conversion of NA to A could augment sympathetic effects on immune cells.

The additional acute immobilization of chronically stressed animals activates the sympatho-adrenomedullary system and increases the synthesis of splenic PNMT protein catalyzing the conversion of NA to A, both of which can modulate the immune functions.

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REFERENCES

Andreassi, J.L., Eggleston, W.B., and *J.K. Stewart* (1998). Phenylethanolamine N –methyltransferase mRNA in rat spleen and thymus. *Neurosci Lett.* **241**, 75-78.

*Fleshner, M. (*2000). Exercise and neuroendocrine regulation of antibody production: protective effect of physical activity on stress-induced suppression of the specific antibody response. *Int J Sports Med.* **21**, 14-19.

Greenwood, B.N., Kennedy, S., Smith, T.P., Campeau, S., Day, H.E., and *M. Fleshner* (2003).Voluntary freewheel running selectively modulates catecholamine content in peripheral tissue and c-Fos expression in the central sympathetic circuit following exposure to uncontrollable stress in rats. *Neuroscience* **120(1)**, 269-81.

Jetschmann, J., Benschop, R.J., Jacobs, R., Kemper, A., Oberbeck, R., Schmidt R.E. and *M. Schedlowski* (1997). Expression and in vivo modulation of alpha and β-adrenoceptors on human natural killer (CD16+) cells. *J. Neuroimmunol.* **74,** 159-164.

Kvetnansky, R. and *L. Mikulaj* (1970). Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. *Endocrinology* **87**, 738-743.

Kvetnansky, R., Sabban, E.L. and *M. Palkovits* (2009). Catecholaminergic Systems in Stress: Structural and Molecular Genetic. *Approaches Physiol Rev*. **89**, 535-606.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

Lands, A.M., Luduerra F.P. and *H.J. Suzzo* (1967). Differentiation of receptors responsive to isoproterenol. *Life Sci*. **6,** 2241-2249

Livak, K.J. and *T.D. Schmittgen* (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)). *Method. Methods* **25**, 402-408.

Peuler, J.D. and *A. Johnson* (1977). Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine. *Life Sci*. **21**, 625-636.

Sanders, V.M., Baker, R.A., Ramer-Quinn, D.S., Kasprowicz, D.J., Fuchs, B.A., and *N.E. Street* (1997). Differential expression of the beta 2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. *J. Immunol*. **158**, 4200-4210.

Shao, F., Lin, W., Wang. W., Washington, W.C., and *L. Zheng* (2003). The effect of emotional stress on the primary humoral immunity of rats. *J. Psychopharmacol*. **17**, 179-183.

Stich, T.M. (1990). Determination of protein covalently bound to agarose supports using bicinchoninic acid. *Annals of Biochemistry*. **191**, 343-346.

Warthan, M.D., Freeman, J.G., Loesser, K.E., Lewis, C.W., Hong, M., Conway, C.M., and *J.K. Stewart* (2002). Phenylethanolamine N-methyltransferase expression in mouse thymus and spleen. *Brain, Behav Immun.* **16**, 493-499.

Weiss, I.C., Pryce, C.R., Jongen-Rêlo, A.L., Nanz-Bahr, N.I., and *J. Feldon* (2004). Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. *Behav Brain Res.* **9 152(2)**, 279-95.

Wong, D.L. and *A.W. Tank* (2007). Stress-induced catecholaminergic function: transcriptional and post-transcriptional control. *Stress* **10(2)**, 121-30.